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PRINCIPAL INVESTIGATOR: Samuel R. Denmeade, M.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, Maryland 21205-2196

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Samuel R. Denmeade, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Johns Hopkins University
Baltimore, Maryland 21205-2196

E-MAIL:
denmeade@mail.jhmi.edu

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13. ABSTRACT *(Maximum 200 Words)*

The aim of this proposal is to develop a method to target novel cytotoxic agents specifically to sites of metastatic prostate cancer. In the original proposal, proteasome inhibitors were selected as the cytotoxic agent. Initial studies revealed that this approach was not tenable due to the inherent instability of these compounds. An alternative agent was then selected to continue this targeted prodrug approach. Thapsigargin (TG) induces apoptosis in a proliferation independent manner in prostate cancer cells. This cytotoxicity, however, is not prostate cell type specific and TG could not be given systemically without significant toxicity. To achieve targeted cytotoxicity the TG analogs will be converted to inactive prodrugs by coupling to a peptide carrier that is a substrate for the serine protease activity of Prostate-Specific Antigen (PSA). Since PSA is expressed in high levels only by normal and malignant prostate cells, this approach should allow specific targeting of the killing ability of TG to prostate cancer cells. Therefore a series of amine containing TG analogs have been synthesized and characterized for their ability to induce apoptosis in prostate cancer cell lines. The lead TG analog has been chemically linked via a peptide bond to a previously identified PSA-specific peptide (i.e. 6 amino acids) to produce an inactive prodrug. This prodrug can be hydrolyzed by PSA and a 25-fold increase in toxicity is seen in the presence of enzymatically active PSA. In vivo studies using this lead TG prodrug to treat PSA-producing human prostate cancers are underway. Additional prodrugs will also be synthesized in order to optimize PSA-targeting.

14. SUBJECT TERMS
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Table of Contents

(4) INTRODUCTION:

Approximately forty thousand American men die annually from metastatic prostate cancer. Standard chemotherapeutic agents have been ineffective at significantly prolonging the survival of men with metastatic prostate cancer and these agents are typically associated with often severe, dose-limiting side effects. New agents are therefore urgently needed. While a large number of cytotoxic agents have been demonstrated to be effective in vitro, these agents are typically general cytotoxins that cannot be administered to patients without severe systemic toxicities. Therefore, what is required is a method to target the delivery of novel, effective cytotoxic agents specifically to sites of metastatic prostate cancer. Such an approach would result in increased concentration of drug within the tumor while avoiding significant systemic toxicity. One such novel agent that has been demonstrated in recent studies to induce apoptosis in a variety of cell types, including prostate cancers, is the natural product thapsigargin (TG). TG potently inhibits the Endoplasmic Reticulum Ca^{2+}-ATPase pump causing a sustained elevation of intracellular calcium that leads to induction of apoptosis in TG-treated cells. The cytotoxicity of TG, however, is not prostate cancer specific. In this proposal, a prostate cancer specific targeting strategy is outlined that will overcome this limitation. To achieve targeted cytotoxicity a potent TG analog will be converted to an inactive prodrug by coupling to a peptide carrier such that the analog can be efficiently converted back to an active killing agent only upon proteolysis by the serine protease activity of a unique prostate-specific protein, Prostate-Specific Antigen (PSA). Since PSA is expressed in high levels only by normal and malignant prostate cells and not in any significant amounts by other normal cell types, this approach should allow specific targeting of the killing ability of TG to prostate cancer cells. Therefore a series of primary amine containing TG analogs will be synthesized and characterized for their ability to induce apoptosis in prostate cancer cell lines and normal fibroblasts. Cytotoxic primary amine containing TG analogs will be chemically linked via a peptide bond to a previously identified PSA-specific peptide (i.e. 6 amino acids) to produce inactive prodrugs. Prodrugs in which the active TG analog can be efficiently released by the proteolytic activity of PSA will be tested for their potency and selectivity as PSA activated killing agents against PSA-producing, androgen independent human prostate cancer cells. The lead prodrug (i.e. the prodrug most efficiently and specifically hydrolyzed by PSA to release most active TG analog) will then be tested in vivo for activity in mice bearing PSA-producing human prostate cancers. These studies will serve to identify the best candidate prodrug that will subsequently tested in clinical trials as treatment for metastatic prostate cancer.

(5) BODY:

The hypothesis of this proposal was that the proteolytic activity of PSA, which is highly expressed by androgen independent prostate cancer cells, can be used to activate prodrugs specifically to cytotoxic metabolites at sites of metastatic prostate cancer. Originally, the specific cytotoxic agents to be targeted were proteasome inhibitors. The original plan of the Award proposal was to develop prodrugs consisting of a proteasome inhibitor coupled to a peptide. The peptide was designed to be a specific substrate for the proteolytic activity of prostate-specific antigen (PSA). In this approach the inactive proteasome inhibitor/peptide prodrug could be given systemically without significant toxicity because PSA is enzymatically active within the blood due to complex formation with serum protease inhibitors. The extracellular fluid of prostate cancers contains large amounts (i.e. mg/ml) of enzymatically active PSA capable of releasing the active drug and inducing apoptosis of the surrounding cells.

The first two tasks of the original proposal were as follows:

Task 1: Synthesis of amine containing proteasome inhibitors and characterization of proteasome inhibition and cytotoxicity (1-12 months).

Task 2: Prodrug synthesis and determination of rates of PSA proteolysis of proteasome inhibitor prodrugs (12-24 months).

For task 1, several amine containing proteasome inhibitors were synthesized (table 1) in collaboration with chemists at Cephalon, Inc. For these inhibitors a primary amine was incorporated into the structure of the inhibitor to allow for coupling to a peptide. These inhibitors were characterized for their ability to inhibit the proteasome in a broken cell proteasome assay, table 1. These compounds demonstrated potent inhibition of the proteasome at low nanomolar concentrations. In whole cell cytotoxicity assays, these compounds were far less potent with IC_{50} values for inhibition of cell growth in the micromolar range (i.e. 1000-fold less potent), table 1.

For task 2, the proteasome inhibitor 6481 (table 1) was coupled to the PSA-specific peptide carrier Acetyl-His-Ser-Ser-Lys-Leu-Gln (Ac-HSSKLQ). This produced a compound that was still capable of potently inhibiting the proteasome in broken cell preparations with an IC_{50} value of 3 nM but demonstrated no cellular cytotoxicity at doses up to 50 μM. This prodrug was then incubated with enzymatically active PSA and assayed for hydrolysis by HPLC analysis. No demonstrable hydrolysis of the prodrug was observed (data not shown). In addition, HPLC analysis demonstrated that this proteasome inhibitor prodrug compound was unstable and rapidly degraded. The possible mechanism for this degradation is a reaction between the epsilon-primary amine of lysine with the boronic ester active group in the proteasome inhibitor. HPLC analysis also demonstrated instability of the uncoupled proteasome inhibitor 6481 by a potentially similar mechanism. Alternatively, the boronic ester group may not be stable in aqueous environment.

Originally, Cephalon Inc. had agreed to supply my laboratory with a series of modified proteasome inhibitors. This company also agreed to help with the synthesis of proteasome inhibitor-peptide prodrugs. However, on the basis of these preliminary unfavorable results, Cephalon Inc. did not wish to continue collaboration on this project. The company sited financial constraints and made a decision not to put any more of their already limited funds into the proteasome project.

These preliminary data obtained from work outlined in task 1 and 2 of the original application suggest that these proteasome inhibitors may not be ideal candidates for coupling to a peptide carrier due to their inherent instability and poor cell penetration. Instead of abandoning the project at this point, I chose to continue the work using an alternative cytotoxic agent. This decision was based on the previous findings in my laboratory suggesting that the defined PSA-specific peptide carrier could be used to effectively target a chemotherapeutic agent to sites of PSA-producing prostate cancer. In this original work, a doxorubicin

analog was coupled to the HSSKLQ peptide carrier to produce a prodrug that was stable and inactive in the absence of enzymatically active PSA (Appendix 1). However, in the presence of active PSA, the cytotoxic doxorubicin analog is released and cells underwent apoptosis. These preliminary studies with the doxorubicin prodrug have provided the rationale for further development of this PSA-based targeting strategy. In recently published work sponsored by this award, a PSA-doxorubicin prodrug was tested in vivo against a PSA-producing human prostate cancer xenograft (Appendix 2) . This prodrug was found to be non-toxic to the treated animals and significantly inhibited the rate of tumor growth. Doxorubicin has been tested previously as treatment for metastatic prostate cancer and, although partial responses were seen in some studies, this agent was not thought to be very effective therapy. Therefore, although doxorubicin may not be the preferred agent, other highly potent, novel cytotoxic agents could be employed in a similar PSA-targeted approach.

The original hypothesis of the proposal was that the proteolytic activity of PSA, which is highly expressed by androgen independent prostate cancer cells, could be used to activate prodrugs specifically to liberate a cytotoxic agent at sites of metastatic prostate cancer. An example of one such cytotoxic agent is the natural plant product thapsigargin (TG) that has been demonstrated in recent studies to induce apoptosis in a variety of cell types, including prostate cancers, figure 1. TG has the unique ability to induce apoptosis in a proliferation independent manner. Therefore, it may be an ideal agent to treat slowly proliferating metastatic prostate cancer. TG would be difficult to administer systemically because its cytotoxicity is not prostate cancer specific and it is also able to kill G_0 arrested cells To achieve targeted cytotoxicity, TG analogs can also be converted to inactive prodrugs by coupling to a peptide carrier such that they can only be efficiently converted back to active killing agents only upon proteolysis by PSA.

Thapsigargin Background

Thapsigargin (TG) is a sesquiterpene g-lactone isolated from the root of the umbelliferous plant, *Thapsia garganica*. TG has been shown to increase intracellular Ca^{2+} and induce programmed cell death in prostate cancer cell lines as well as a host of other normal and malignant cell types. More recent studies have shown that TG inhibits the sarcoplasmic/endoplasmic reticulum (ER) Ca^{2+}-ATPase (SERCA) pump with an IC_{50} value of 30 nM. This inhibition is not only efficient but also highly specific, since neither plasma membrane nor red blood cell Ca^{2+}-ATPase is inhibited, even at micromolar concentrations of TG. Large pools of bound calcium are sequestered in the ER of cells even thought the $(Ca)_i$ concentration is only 30 to 40 nM. In response to a variety of intracellular signals, such as inositol 1,4,5-triphosphate (IP3), an elevation of intracellular Ca^{2+} $(Ca)_i$ to several hundred nMs occurs. The elevation is usually transitory, however; the Ca^{2+} is rapidly pumped out of cells via the plasma membrane Ca^{2+}-ATPase pumps or back into the ER via its SERCA pump. Furthermore, the ER-sequestered Ca^{2+} is constantly leaking out into the cytoplasm. The sequestered store of Ca^{2+} is constantly replenished, however, by the SERCA pumps ability to transport the cytoplasmic Ca^{2+} back into the ER. Thus, inhibition of the SERCA pumps results in a threefold to fourfold elevation of $(Ca)_i$ (without any requirement for IP3 production). This primary elevation of $(Ca)_i$ leads to a depletion of the ER-Ca^{2+} pool and, in many cell types, results in generation of a signal that induces a change in the plasma membrane permeability to extracellular divalent cations, particularly Ca^{2+} . The initial intracellular discharge of the ER-sequestered calcium pools leads to an influx of extracellular Ca^{2+}, in keeping with the prediction from the capacitance model of Ca^{2+} entry, resulting in a secondary elevation in the $(Ca)_i$ and to activate programmed cell death in androgen-independent prostate cancer cells.

In cells treated with the TG bi-phasic changes in intracellular free calcium have been observed. After an initial increase from baseline values of 20-40 nM to values of 200-400 nM, induced by the emptying of the endoplasmic reticulum calcium pool, intracellular calcium return to baseline values within 6 to 18 hours. This decrease is mediated by activation of the calmodulin-dependent calcium pump of the plasma membrane since cells microinjected with a calmodulin inhibiting peptide maintained an elevated calcium . This first rise can be attenuated by intracellular buffers such as BAPTA or the calcium binding protein calbindin. In all

dying cells a sustained second elevation of intracellular free calcium from a baseline of 20 -40 nM to 10- 50 μM has been observed. This rise to μmolar values precedes the morphological changes associated with apoptosis in both prostate and breast cancer cells. This second rise is asynchronous within the cell population but ultimately occurred in every dying cell. The proportion of cells showing a second increased per unit of time correlates with the number of cells showing DNA fragmentation and the proportion of cells showing loss of viability when measured by clonogenic assay. These results demonstrate the critical role of sustained elevations of intracellular Ca2+ in the programmed cell death induced by TG.

Chronic exposure of each cancer cell line to 500 nM TG was found to arrest the cells in the G_0/G_1 phase of the cell cycle within 24 hours. Analysis of mRNA expression of genes previously shown to be enhanced during androgen ablation-induced programmed cell death of normal prostate cells (e.g. calmodulin, TRPM-2, etc) showed that TG treatment of androgen-independent prostate cancer cells also leads to epigenetic reprogramming of the cells. Within 1 hour of TG treatment, androgen-independent cancer cells had elevated expression of additional genes, including glucose-regulated protein (GRP)B78, c-myc, and growth arrest and DNA damage (GADD)-153. Many of these enhancements were acute, with expression decreasing by 24 hours of treatment. After a 24-hour lag period, the cells began to fragment their DNA (to sizes <300 kb); by 96 hours, 95% or more had fragmented their DNA regardless of cell line tested. Quantitative analysis of the DNA showed the nucleosomal ladder pattern of fragmentation characteristic of programmed cell death.

Prostate cancer cells must progress through the proliferative cell cycle in order for antiproliferative agents such as 5-fluorodeoxyuridine to induce programmed cell death, whereas proliferation is not required for TG-induced apoptosis. Furthermore, there are major differences in gene expression during the proliferation-independent programmed death induced by TG and the proliferation-dependent apoptosis associated with 5-FrdU. In additional studies, primary cultures of human prostate cancer cells were made. These cultures initially grow exponentially. During this exponential phase treatment with equal concentrations of either the cell proliferation-dependent chemotherapeutic agents 5-FrdU or doxorubicin or thapsigargin resulted in sterilization of culture dishes. In contrast, these cells were shown to go out of cycle and enter the proliferatively quiescent G_0 state after an initial proliferation period of approximately 10 days. The cultures were maintained for more than 6 weeks, with a spontaneous rate of cell death of ~2% /day. When these stationary cultures were exposed for 1 week to effective doses (i.e.100 nM) of doxorubicin or 5-FrdU, there was no significant activation of PCD either morphologically by videomicroscopic evaluation or quantitatively by DNA fragmentation . In contrast, exposure of the stationary cultures to 100 nM TG resulted in morphologic changes within 24 to 48 hours, with loss of ~ 85% of cells by day 4 of exposure. Thus, TG can induce programmed cell death of proliferatively quiescent G0 human prostate cancer cells without requiring their entry into or progression through the cell cycle.

Preliminary Data with TG Analogs

On the basis of these preclinical studies, it would appear that TG represents an excellent choice for treatment of prostate cancer because of its ability to kill prostate cancer cells in a proliferation-independent manner. Unfortunately, while TG is highly effective in inducing the proliferation independent programmed cell death of androgen independent prostate cancer cells, it is not cell type specific and is sparingly water soluble due to its high lipophilicity. In order to target TG's cytotoxicity specifically to prostate cancer cells systemically, TG must be chemically modified to produce an analog that can be coupled to a water-soluble prodrug carrier. This modification involves the introduction of a primary amine containing side chain into the TG molecule that can be coupled via a peptide bond to the carboxyl group of the C-terminal amino acid. In this way, TG can be targeted specifically to metastatic deposits of androgen independent prostate cancer producing enzymatically active PSA.

As a fellow in Dr. Isaacs laboratory I began collaboration with Dr. S. Brogger Christensen, Professor of Medicinal Chemistry at the Royal Danish School of Pharmacy, Copenhagen, Denmark. Dr. Christensen

originally isolated and chemically characterized TG. Based on a model of the TG binding site within the SERCA pump it was determined that modifications of the TG molecule could possibly be made in the side chain in the 8-position without adversely effecting SERCA pump inhibitory activity. These modifications consist of de-esterifying TG in a position 8 and re-esterifying with side chains ending in a primary amine to allow for coupling via an amide bond to the C-terminal carboxylic acid of the PSA hydrolyzable peptide carrier, HSSKLQ. Using this rationale a series of TG analogs (i.e. ~30) modified in the 8-position with primary amine containing side chains were synthesized (Appendix 3). These analogs were characterized for their ability to inhibit the SERCA pump and elevate intracellular calcium. In addition, these analogs were assayed for cytotoxic activity against androgen independent human prostate cancer cells in vitro.

From these studies, an initial lead TG analog, termed APT, was identified with an LC50 of 275 nM against the human prostate cancer cell line TSU-PR. After characterizing the cytotoxicity of the APT analog, the HSSKLQ-APT prodrug was synthesized. Hydrolysis by PSA did not occur when the APT analog was directly coupled to the carrier peptide. A spacer or linker consisting of the amino acid leucine had to be introduced before PSA hydrolysis was observed. The introduction of this linker resulted in a TG analog with an LC_{50} value of 1 μM (i.e. approximately 4 times less active than APT and 30 times less active than TG), (figure 2 and 3). A small amount of the prodrug HSSKLQ-Leu-APT was then synthesized. HPLC analysis demonstrated that PSA could liberate Leu-APT from the peptide. In cytotoxicity assays in vitro, the LC_{50} against PSA-producing LNCaP cells was approximately 500 nM. In addition, against PSA non-producing TSU-Prl cells the LC50 of the prodrug was approximately 20 μM while against TSU cells growing in media the LC50 was ~2μM. Thus there was a 10-fold difference in the therapeutic efficacy when this prototype prodrug was administered in the presence of PSA. These in vitro studies demonstrated that PSA could effectively hydrolyze the HSSKLQ-Leu-APT prodrug to produce effective cytotoxic levels of Leu-APT.

Progress During the Previous 12 month Funding Period

When it became clear that the approach using proteasome inhibitors was no longer viable due to lack of support from Cephalon Inc. I used the support from the New Investigator Award has allowed me to extend the earlier work on TG analogs that I began as a fellow. The ultimate goal of this work is to; (1) identify and characterize a TG analog with cytotoxicity approaching that of TG in clonogenic survival assays; (2) from these analogs identify one that can be coupled to the PSA-specific peptide and be efficiently hydrolyzed by enzymatically active PSA.

Over the past year, with support from the New Investigator Award, a series of additional primary amine containing TG analogs were synthesized and assayed for activity against human prostate cancer cell lines. These analogs contained long hydrocarbon side chains and ended in a primary amine (figure 1). The rationale for the design of these analogs was that the longer linker would keep the large TG molecule out f the PSA catalytic site. One of theses analogs, containing a 12-amino dodecanoate side chain (12ADT) was found to have an IC_{50} value against TSU cells of ~ 500 nM (figure 2). This analog was coupled to the PSA-peptide carrier and once again no hydrolysis was observed when incubated with enzymatically active PSA.

From my earlier work with the doxorubicin prodrugs and the more recent results with the TG analog prodrugs, it was apparent that an amino acid was required in the P_{-1} position of the prodrug (i.e. HSSKLQ-P_{-1}AA). When the P_{-1} amino acid was leucine, PSA-hydrolysis was observed with both doxorubicin and the APT analog. Therefore, leucine was coupled to several TG analogs. In this way, a TG analog consisting of leucine coupled to a 12-carbon (i.e. aminododecanoic acid) side chain was produced [i.e. Leu-aminododecanoic-TG (L-12ADT)] (figure 2). The rationale for this compound was that the increase in lipophilicity secondary to the long 12-carbon side chain would increase plasma membrane permeability to compensate for the positively charged leucine. The L-12ADT analog has an IC50 value of 75 +/- 5 nM against TSU cells in clonogenic survival assays (figure 2). This level of activity was similar to the parent TG, making this the most potent TG analog developed to date. The L-12ADT analog was 20 fold more potent than the Leu-APT analog.

On the basis of this excellent cytotoxic profile, the L-12ADT was coupled to the HSSKLQ peptide carrier. HPLC analysis demonstrated that PSA could liberate L-12ADT from the peptide with ~50% conversion in 24 hrs (figure 3). In cytotoxicity assays in vitro, the LC_{50} against PSA-producing LNCaP cells was 100 nM. In addition, against TSU cells the LC_{50} was ~150 nM when enzymatically active PSA was present in the media and ~3.5 μM when PSA was not present (figure 4). Thus there was a ~25-35 fold difference in therapeutic efficacy when this prodrug was administered in the presence or absence of PSA.

Proposed Studies for the 2001 Funding Period

On the basis of these exciting results with the HSSKLQ-L12ADT prodrug, further studies are warranted. In order to continue on the timeline outlined in the original Statement of Work I intend to synthesize a series of additional peptide-TG analogs in which the amino acid linker is changed to a different amino acid or to D-Leucine. These additional analogs will be tested for cytotoxicity in vitro against the human prostate cancer cell line TSU. These analogs will also be coupled to the HSSKLQ peptide carrier and assayed for PSA hydrolysis as outlined in Task 3 of the original proposal. The 12-ADT analog, which is not as potent a cytotoxin as L-12ADT, will also be directly coupled to the peptide and assayed for PSA hydrolysis. In this way the optimal lead prodrug will be defined consisting of a TG analog that is both a potent cytotoxin and efficiently hydrolyzed from the peptide carrier by PSA.

Additionally, over the next year, sufficient amounts of this lead prodrug will be synthesized to begin animal studies as outlined in task 4 of the original proposal. Nude mice will be inoculated either subcutaneously or orthotopically with the PSA-producing human prostate cancer cell line LNCaP. These studies will determine antitumor efficacy of the lead prodrug as well as define host toxicity. Once antitumor effect is demonstrated, further studies will be initiated to determine the optimal route of delivery and dosing regimen of this prodrug. Radiolabeled prodrug will be synthesized in order to study distribution and metabolism of the prodrug by normal tissues and within tumors.

(6) KEY RESEARCH ACCOMPLISHMENTS:

1. Synthesized primary amine containing proteasome inhibitors that inhibit proteasome at low nM concentrations.

2. Demonstrated that these inhibitors are unstable in solution and when coupled to peptides.

3. Characterized a series of primary amine containing analogs of the cytotoxic natural product thapsigargin (TG).

4. Identified a TG analog containing the amino acid linker leucine that is a highly potent cytotoxin that induces apoptosis at low nanomolar concentrations.

5. Demonstrated that this leucine containing TG analog (L-12ADT) can be coupled to a PSA-specific peptide and be hydrolyzed free from the peptide by enzymatically active PSA.

6. Demonstrated that this prodrug is relatively inactive against PSA non-producing cancer cells while ~ 30 fold enhancement of therapeutic effect occurs in the presence of PSA.

(7) REPORTABLE OUTCOMES:

Manuscripts, abstracts, presentations;

1. Khan SR, **Denmeade SR**. In Vivo Activity of a PSA-Activated Doxorubicin Prodrug Against PSA-Producing Human Prostate Cancer Xenografts. Prostate 45:80-83, 2000.

2. **Denmeade SR**, Jakobsen CM, Khan SR, Gady AM, Christensen SB, Isaacs JT. Enzymatic activation of a thapsigargin prodrug by prostate specific antigen (PSA) as treatment for metastatic prostate cancer. Proc. Am. Assoc. Cancer Res. 41:46 #292, 2000. (Abstract).

Patents and Licenses applied for and/or issued;

1. "Tissue Specific Prodrug", Inventors: Isaacs, JT, **Denmeade, SR**, Christensen, SB, Lilja H. International Patent Application PCT/US98/10285, filed 5/19/97 and 3/30/98. This patent refers to the PSA-specific peptide, the creation of PSA targeted prodrugs, and primary amine containing TG analogs.

2. "Activation of Peptide Prodrugs by Human Glandular Kallikrein 2 (hK2)", Inventors: Isaacs, JT, **Denmeade, SR**, Lilja H. Patent filed 7/00. This invention refers to development of peptide substrates for the protease hK2, the creation of hK2 targeted prodrugs, and also refers specifically to the TG analog L-12ADT.

Funding applied for based on work supported by this award;

1. Clinician Scientist Award from Johns Hopkins School of Medicine (Two year salary support for Dr. Samuel Denmeade).

(8) CONCLUSIONS:

The original hypothesis of the proposal was that the proteolytic activity of PSA, which is highly expressed by androgen independent prostate cancer cells, could be used to activate prodrugs specifically to liberate a cytotoxic agent at sites of metastatic prostate cancer. In the original proposal, I had intended to use proteasome inhibitors as the preferred cytotoxic agent. Preliminary studies demonstrated that this approach might not be feasible due to instability of the proteasome inhibitors and poor cell penetration. In addition, the pharmaceutical company that I was collaborating with decided not to continue this work, citing lack of funds.

Instead of abandoning the project at that early point, I decided to continue the project and use a different cytotoxic warhead by substituting a TG analog for the proteasome inhibitor. In doing so, I was building on the work with TG that I began as a post-doctoral fellow. TG represents an ideal treatment for slowly proliferating prostate cancers as it can induce apoptosis in a proliferation independent manner. To develop the TG-based prodrug I have followed the same task list and timetable as that outlined in the original proposal. For the renewal year I intend to complete tasks 3 and begin task 4 of the original proposal, substituting TG analogs for proteasome inhibitors as the cytotoxic agent that will be coupled to the PSA-specific peptide.

The preclinical data with the TG analog L12ADT (figure 2,3) demonstrate that it is a potent cytotoxin against prostate cancer cell lines. When coupled to the PSA-specific peptide, the prodrug HSSKLQ-L12ADT is readily hydrolyzed by PSA. This prodrug is relatively inactive in vitro in the absence of enzymatically active PSA in the media; however, when PSA is present the activity increases ~ 30 fold. These results demonstrate the feasibility of the TG-prodrug approach and indicate that further development of TG-prodrugs is warranted.

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(10) Appendices

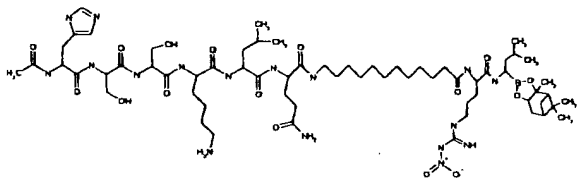
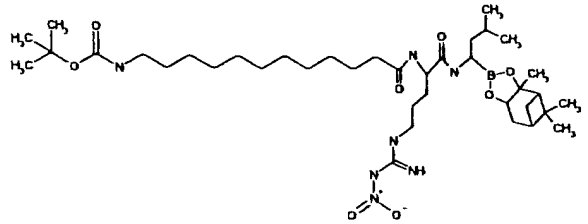
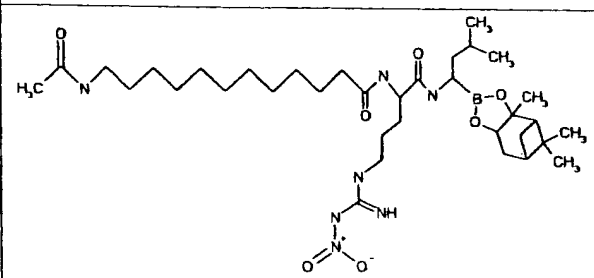
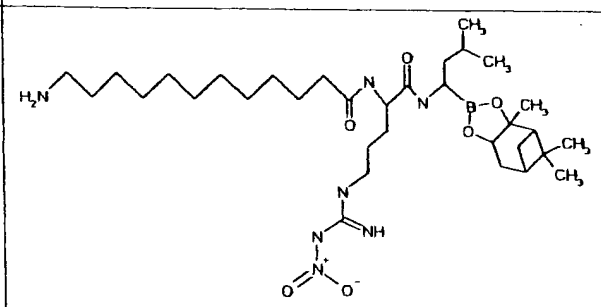
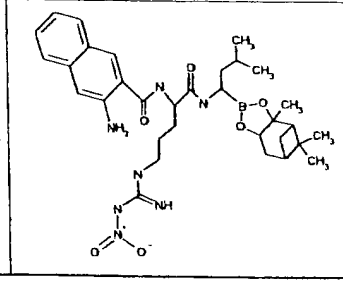
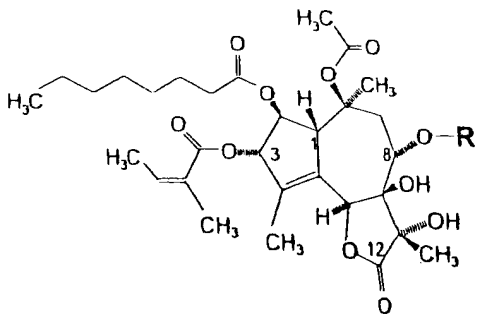
Cep #	Structure	Proteasome IC_{50} (nM)	Cell IC_{50} (uM)
0006586		3	No Inhibition
0006478		2	0.2
0006484		3	1
0006481		4	9
0006081		8	2

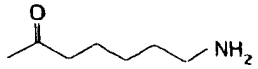
Table 1. Structure of primary-amine containing proteasome inhibitors and corresponding IC_{50} values for proteasome inhibition and toxicity to TSU prostate cancer cells.

Figure 1

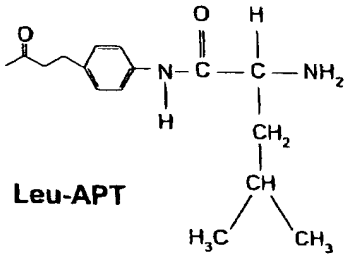


R=

Thapsigargin (TG)



6AHT



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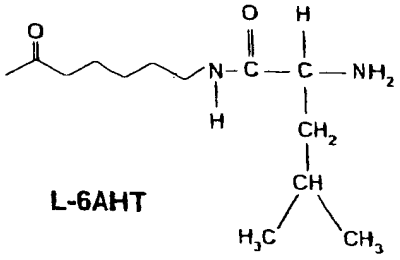

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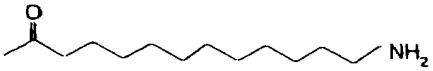
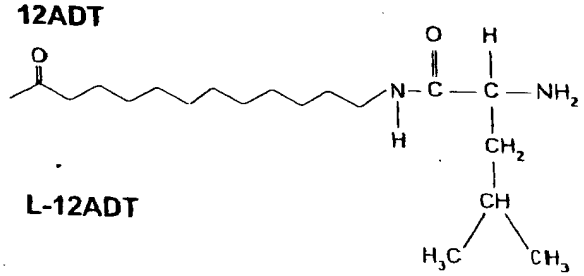


12ADT



L-12ADT

Figure 2

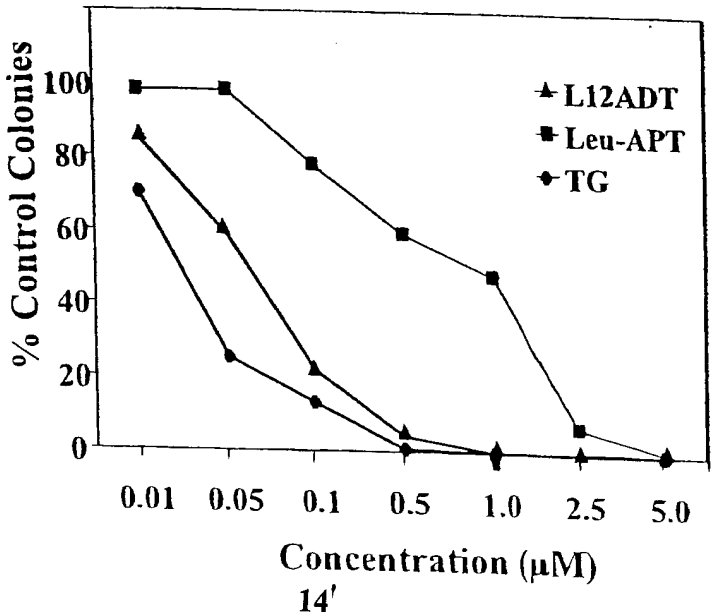


Figure 3.

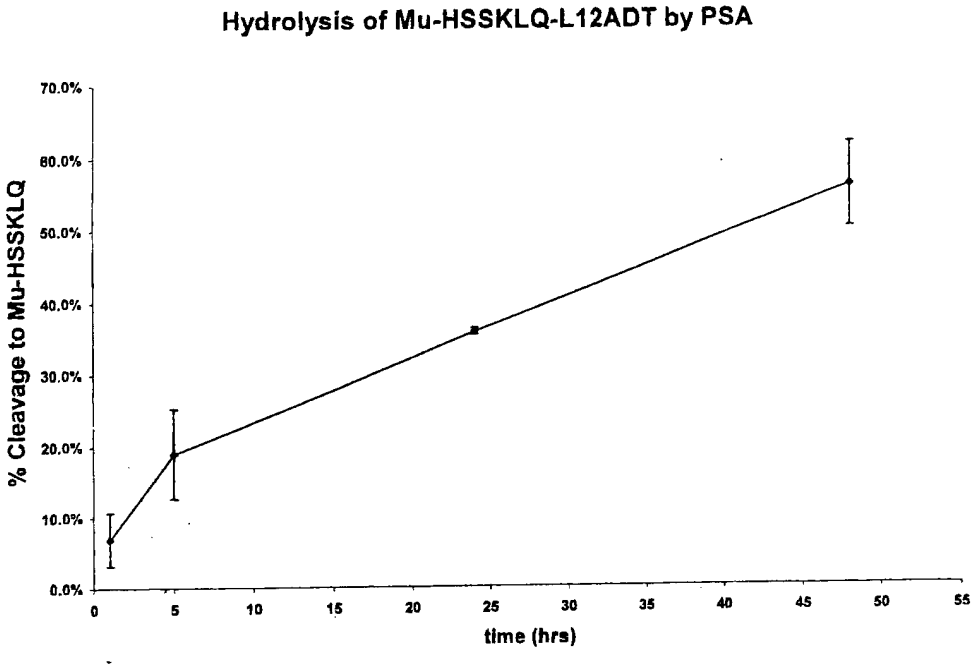
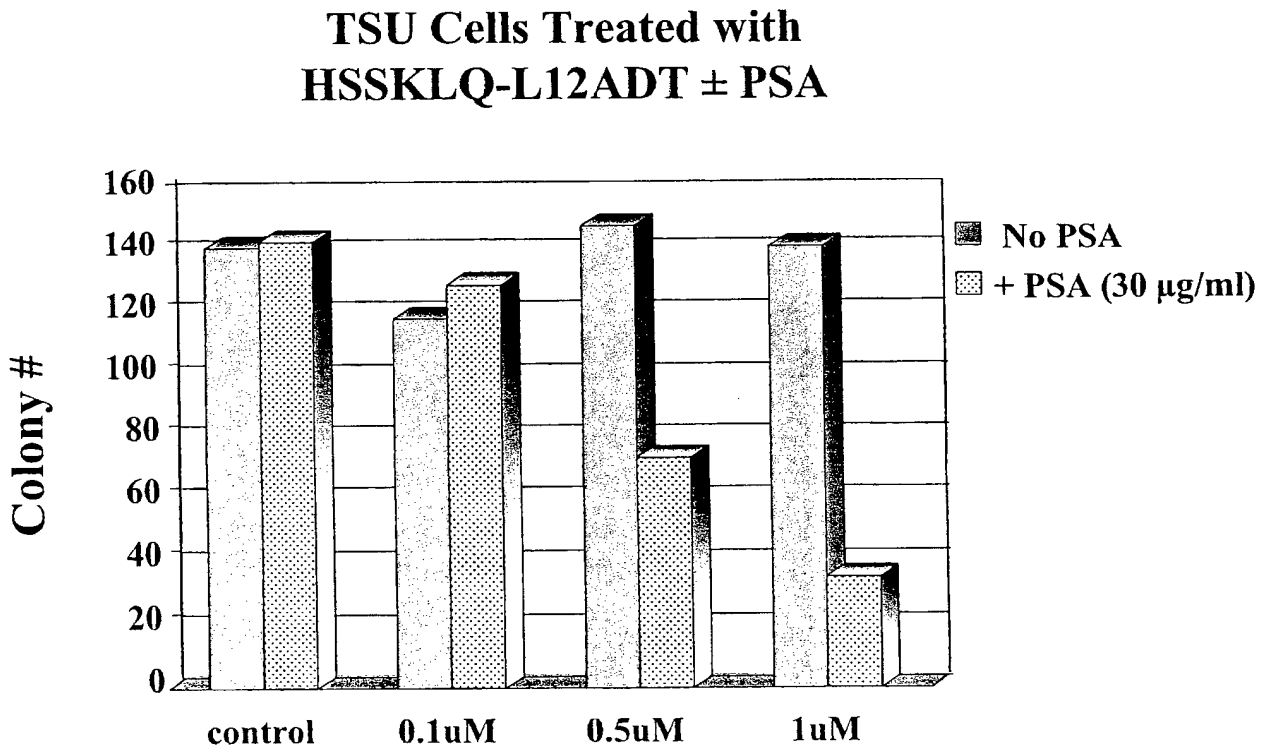


Figure 4.



[CANCER RESEARCH 58, 2537–2540, June 15, 1998]

Advances in Brief

Enzymatic Activation of a Doxorubicin-Peptide Prodrug by Prostate-Specific Antigen[1]

Samuel R. Denmeade, Attila Nagy, Jin Gao, Hans Lilja, Andrew V. Schally, and John T. Isaacs[2]

Johns Hopkins Oncology Center [S. R. D., J. G., J. T. I.] and James Buchanan Brady Urological Institute [J. T. I.], Johns Hopkins School of Medicine, Baltimore, Maryland 21231-1001; Department of Medicine, Tulane University School of Medicine and Veterans Affairs Medical Center, New Orleans, Louisiana 70112 [A. N., A. V. S.]; and Department of Clinical Chemistry, Lund University, University Hospital, Malmö S-214 01, Sweden [H. L.]

Abstract

New approaches to target cytotoxic therapy specifically to metastatic prostate cancer sites are urgently needed. As such an approach, an inactive prodrug was synthesized by coupling the primary amine of doxorubicin to the COOH-terminal carboxyl of a seven-amino acid peptide carrier (*i.e.*, Mu-His-Ser-Ser-Lys-Leu-Gln-Leu). The seven-amino acid peptide was documented to be hydrolyzable specifically by the serine protease prostate-specific antigen (PSA) to liberate the active cytotoxin L-leucyl-doxorubicin. Primary cultures of PC-82 human prostate cancer cells secreted high levels of enzymatically active PSA (*i.e.*, 70 ± 5 ng of enzymatically active PSA/10^6 cells/24 h), whereas LNCaP human prostate cancer cells produced lower levels of enzymatically active PSA (*i.e.*, 2.3 ± 1 ng/10^6 cells/24 h). LNCaP cells, however, secreted sufficient amounts of enzymatically active PSA to activate the doxorubicin prodrug to a cytotoxic form *in vitro*. The specificity of the cytotoxic response to the prodrug was demonstrated by the fact that 70 nM of the prodrug killed 50% of the PSA-producing LNCaP cells, whereas doses as high as 1 μM had no cytotoxic effect on PSA-nonproducing TSU human prostate cancer cells *in vitro*.

Introduction

There is currently no effective therapy for men with metastatic prostate cancer with relapse after androgen ablation, even though numerous agents have been tested over the past 30 years (1). Prolonged administration of effective concentrations of standard chemotherapeutic agents is usually not possible because of dose-limiting systemic toxicities. Therefore, new strategies are needed to target cytotoxic agents specifically to sites of metastatic prostate cancer while avoiding systemic toxicity. One such approach would be to develop prodrugs that are inactive when given systemically but become activated when processed proteolytically within prostate cancer metastases. To accomplish this, primary amine-containing agents can be coupled through the amino group to form a peptide bond to a peptide carrier (2).

One such primary amine-containing agent that can be readily modified to a prodrug form is the anthracycline doxorubicin (3). As a single agent, doxorubicin appears to have one of the best response rates among the large number of agents tested in the treatment of prostate cancer (4, 5), but cardiotoxicity and myelotoxicity have limited its use, especially in elderly men. Modifying doxorubicin to an inactive prodrug form may allow prolonged administration with substantially reduced toxicity. To design this prodrug, doxorubicin was linked through its primary amine to a peptide carrier so that the peptide linkage between the cytotoxic agent and the peptide could be specifically cleavable only by the proteolytic activity of PSA.[3] PSA is a M_r 33,000 single-chain glycoprotein that, in men, is synthesized and secreted in large quantities by normal and malignant prostatic epithelial cells (5–7).

PSA is a serine protease with chymotrypsin-like substrate specificity (8–10) that is found in high concentrations (*i.e.*, mg/ml) in the seminal plasma, where the major proteolytic substrates for PSA are the gel-forming proteins in freshly ejaculated semen, Sg I and Sg II (9). On the basis of the PSA cleavage map for Sg I and II, a peptide with the amino acid sequence His-Ser-Ser-Lys-Leu-Gln (HSSKLQ) was identified that had a high degree of specificity for PSA (11). This substrate was used to demonstrate that prostate cancer cells secrete enzymatically active PSA into the extracellular fluid and that PSA becomes inactivated by serum protease inhibitors on entering the blood (11). To study the proteolytic processing of the doxorubicin prodrug by PSA, an *in vitro* assay was developed using the androgen-sensitive human prostate cancer cell line LNCaP, which is the only prostate cancer cell line available that continues to produce PSA on serial passaging (12). In the present study, the enzymatic activity of PSA secreted by LNCaP cells *in vitro* was directly measured using the HSSKLQ-AMC substrate. After characterizing the enzymatic activity of PSA produced by LNCaP cells *in vitro*, doxorubicin prodrugs were synthesized and tested for *in vitro* activity against the LNCaP cell line.

Materials and Methods

Cell Lines. The androgen-responsive LNCaP and androgen-independent TSU-Pr1 human prostate cancer lines were obtained from American Type Culture Collection (Manassas, VA) and maintained by serial passage in RPMI 1640 containing 10% FCS with 100 units/ml penicillin G and 100 units/ml streptomycin sulfate (antibiotics from M. A. Bioproducts, Walkersville, MD) as standard medium in 5% CO_2/95% air at 37°C. The origins and characteristics of the LNCaP and TSU-Pr1 cell lines have been described previously (12, 13). To collect PSA to assay enzymatic activity, LNCaP cells were grown in standard medium to 70–80% confluence. The serum-containing medium was then removed, cells were washed twice with HBSS, and then new serum-free medium was added that consisted of RPMI 1640 containing antibiotics and 100 nM dihydrotestosterone. PC-82 androgen-dependent human prostate cancer xenografts were maintained by serial passage in athymic nude mice (Charles River). The origins and characteristics of this xenograft have been described (14). Primary cultures of PC-82 were established as described (15).

Kinetic Analysis of Substrate Hydrolysis by PSA. Substrate hydrolysis was studied by measuring fluorescence change secondary to AMC release, as described previously (11).

PSA Immunoprecipitation. PSA concentration in conditioned tissue culture medium was determined using the Tandem-R PSA assay (Hybritech, San Diego, CA) according to the manufacturer's instructions. Samples were then diluted to equivalent PSA concentrations in PSA assay buffer. PSA immunoprecipitation was then performed using the mouse monoclonal anti-PSA anti-

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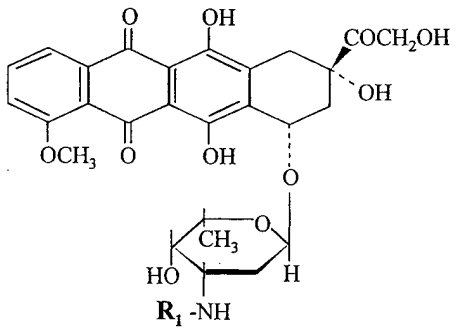
[2] To whom requests for reprints should be addressed, at Johns Hopkins Oncology Center, Johns Hopkins School of Medicine, 422 North Bond Street, Baltimore, MD 21231-1001.

[3] The abbreviations used are: PSA, prostate-specific antigen; Sg, semenogelin; AMC, 7-amino-4-methyl-coumarin; HPLC, high-performance liquid chromatography; mAb, monoclonal antibody; ACT, α_1-antichymotripsin; A2M, α_2-macroglobulin.



When R_1= H- = Doxorubicin (Dox)

When R_1= Ac-His-Ser-Ser-Lys-Leu-Gln- = Ac-HSSKLQ-Dox

When R_1= Mu-His-Ser-Ser-Lys-Leu-Gln-Leu- = Mu-HSSKLQ-Leu-Dox

Fig. 1. Doxorubicin-peptide prodrug structures.

body H117 that recognizes both free PSA and PSA complexed to ACT (16), as described previously (11).

Western Blotting. Samples containing 50 ng of PSA were separated by denaturing SDS-PAGE using 4–20% gradient gels (Bio-Rad) followed by transfer to Hybond-ECL nitrocellulose membranes (Amersham Corp.). Otherwise, the procedure was performed as described previously (11).

Sample Preparation. Conditioned medium from LNCaP cells containing the doxorubicin prodrug were applied to a C_{18} reversed-phase Bond-Elut column (Varian, Carpinteria, CA) and washed with 3 ml of buffer (*i.e.*, six column volumes) consisting of 0.1 M phosphoric acid and 5% acetonitrile in PBS as described (17). Samples were eluted from the column using 2 ml of a solution of 70% acetonitrile/0.1% TFA (v/v). The solvents were then evaporated to dryness, and the samples were redissolved in 0.1% TFA (v/v) and applied to the HPLC column.

Chromatography. The HPLC system consisted of a dual-pump (model 126; Beckman Instruments, Columbia, MD) with a manual injection valve (Rheodyne, Cotati, CA) fitted with a 1-ml injection loop. A reversed-phase C_{18} Ultrasphere analytical column (Beckman) 15 cm × 4.6 mm (inner diameter) was used together with a 4.5 cm × 4.6 mm (inner diameter). Ultrasphere reversed-phase guard column (Beckman). A gradient elution was performed consisting of eluent A [0.1% TFA (v/v)] and eluent B [70% acetonitrile/0.1% TFA (v/v)], with a gradient of 0–30% B over 5 min and then 30–60% B over 25 min, with a flow rate of 1 ml/min. A diode array detector (model 168; Beckman) was used to monitor the effluent at 480 nm. All analyses were conducted at ambient temperature. Data processing was performed using the Gold Chromatography Data System, version 1.0 (Beckman).

Cytotoxicity Assays. Percentage clonogenic survival of TSU-Pr1 (2×10^5 cells) following 48-h exposure to varying concentrations of doxorubicin prodrugs with or without exogenously added PSA was determined as described (18). To analyze the cytotoxicity of the doxorubicin prodrugs against LNCaP cells, these cells were exposed to varying concentrations of prodrugs for 72 h. Cells were then counted, and the percentage viable cells was determined by trypan blue exclusion using a hemocytometer. The dose that produced 50% cytotoxicity as compared to controls was then determined (*i.e.*, the LD_{50}) for both LNCaP and TSU cells.

Materials. The MU-HSSKLQ-AMC substrate was custom synthesized by Enzyme Systems Products (Dublin, CA) and characterized as described (11).

Doxorubicin (Dox) prodrugs [N-Ac-His-Ser-Ser-Lys-Leu-Gln-Dox (Ac-HSSKLQ-Dox), where Ac is acetyl, and His-Ser-Ser-Lys-Leu-Gln-Leu-Dox (Mu-HSSKLQ-Leu-Dox), where Mu is morpholinocarbonyl] were synthesized by coupling the primary amine of doxorubicin to the carboxyl group of the COOH-terminal amino acid [by one of us (A. N.) and California Peptide Research, Inc., Napa, CA; Fig. 1]. Purification of both compounds by HPLC yielded the trifluoroacetate salt (>98% purity). The peptide sequence was confirmed by amino acid analysis, and molecular weights were confirmed by mass spectroscopy.

Doxorubicin was from Pharmacia (Kalamazoo, MI). L-Leucyldoxorubicin was synthesized by A. N. PSA was purified from human seminal plasma as described (19), ACT, A2M, and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Mouse monoclonal IgG anti-PSA antibodies H117 and 5A10 were described previously (16).

Results and Discussion

Enzymatic Activity of PSA Produced by PC-82 Cells *In Vitro*. The PC-82 human prostate cancer xenograft model continues to secrete large quantities of PSA on serial passage in nude mice. PC-82 tumors can be removed and grown as primary *in vitro* cultures in defined serum-free medium containing 10^{-10} M dihydrotestasterones. Medium from these PC-82 primary cultures was collected after 4 days of conditioning, and after concentrating the medium, the PSA concentration was determined using the Tandem R assay. These results demonstrated that PC-82 cells secrete 70 ± 5 ng of PSA/10^6 cells/24 h. This secreted PSA was then assayed for enzymatic activity using the HSSKLQ-AMC substrate and was found to have 99.8 ± 6% of the activity of similar concentrations of enzymatically active PSA purified from human seminal plasma. The enzymatic activity was nearly completely (>98%) inhibited by the PSA-specific mAb 5A10. These results indicate that PC-82 primary cultures secrete PSA in an enzymatically active form and that hydrolysis of the HSSKLQ-AMC was solely due to PSA activity.

Enzymatic Activity of PSA Secreted by LNCaP Cells *in Vitro*. The LNCaP human prostate cancer cell line is androgen sensitive and is the only serially passageable cell line that continues to secrete PSA *in vitro*. Serum-free media were used to eliminate the excess protease inhibitors commonly found in serum that could inactivate any enzymatically active PSA produced by these cells and 100 nM DHT was added to achieve the highest possible yield of PSA. After 4 days of conditioning by the LNCaP cells, the medium was removed and concentrated 20-fold, and the PSA concentration determined using the Tandem R assay. These results demonstrated that under these conditions, LNCaP cells secrete 14 ± 1 ng of PSA/10^6 cells/24 h (*i.e.*, 5-fold less than secreted by PC-82 cells). Initially, these medium concentrates were directly assayed for enzymatic activity using the HSSKLQ-AMC substrate, and no detectable activity was seen. To increase the total amount of PSA in each assay, an additional immunoprecipitation step using the PSA-specific mAb H117 was performed. The specificity and efficiency of this immunoprecipitation has been previously validated using enzymatically active PSA purified from seminal plasma (11). After immunoprecipitation of PSA

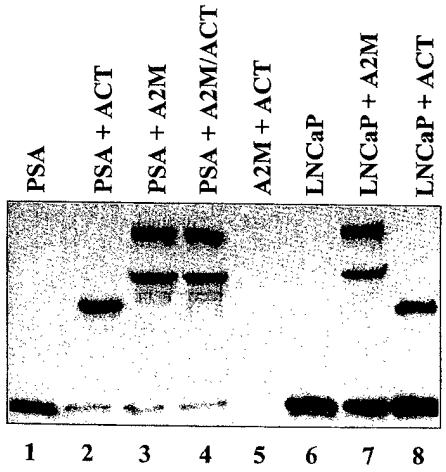


Fig. 2. Western blot analysis of PSA from LNCaP cells with protease inhibitors. *Lanes 1–4*, enzymatically active PSA purified from human seminal plasma incubated with 10-fold molar excess of protease inhibitors ACT and A2M. *Lane 5*, A2M and ACT controls (no PSA). *Lanes 6–8*, LNCaP concentrated PSA-containing medium incubated with indicated protease inhibitors. PSA samples (50 ng of total PSA/*lane* as determined by Tandem R assay) were incubated for 24 h at 37°C prior to analysis. The molecular weight of each band was determined by use of molecular weight marker standards.

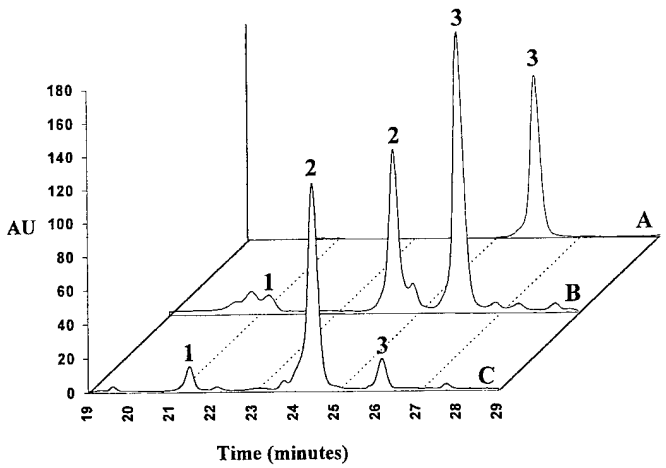


Fig. 3. HPLC analysis of proteolysis of doxorubicin prodrugs and by PSA. *A*, HSSKLQ-Dox, 0.4 mM incubated with 30 μg/ml enzymatically active PSA for 24 h, and then 20 nmol of total doxorubicin were applied to the column. *B*, Mu-HSSKLQ-Leu-Dox, 0.4 mM incubated with 30 μg/ml enzymatically active PSA for 24 h and then 40 nmol of total doxorubicin were applied to the column. *C*, spent medium from LNCaP cells treated with 10 μM Mu-HSSKLQ-Leu-Dox for 72 h, followed by medium extraction as described, and then 20 nmol of total doxorubicin were applied to the column. *Peak 1*, doxorubicin; *peak 2*, Leu-Dox; *peak 3*, doxorubicin-peptide prodrugs. In each case, peaks were verified by use of purified standards.

from PC-82 primary cultures, 95 ± 5% of the immunoprecipitated PSA from these samples retained enzymatic activity.

Following the immunoprecipitation step, the enzymatic activity from a total 1 μg of PSA could be evaluated per assay, and therefore enzymatic activity can be detected, even if only 5% of the total PSA/assay is enzymatically active. When 1 μg of total PSA was immunoprecipitated from the LNCaP-conditioned medium, it possessed only 16 ± 1.5% of the enzymatic activity of similar concentrations of immunoprecipitated PSA purified from seminal plasma (*i.e.*, the LNCaP secreted 2.3 ± 1 ng of enzymatically active PSA/10^6 cells/24 h). Similar to the enzymatic activity of the purified seminal plasma PSA immunoprecipitate, the enzymatic activity of the PSA immunoprecipitated from the LNCaP medium was inhibited by >98% by the neutralizing antibody mAb 5 A10.

To determine whether the PSA secreted by LNCaP cells is inactivated due to the presence of a protease inhibitor in the conditioned medium, enzymatically active PSA purified from human seminal plasma was added to the flask containing LNCaP cells and conditioned medium. The purified PSA was incubated in this way for 24 h and then concentrated and immunoprecipitated as described previously. Using the HSSKLQ-AMC substrate, it was determined that the PSA isolated in this manner maintained 98.6 ± 1% of the enzymatic activity of similar concentrations of immunoprecipitated purified PSA. These results demonstrate that the presence of a large amount of inactive PSA in the conditioned medium from LNCaP cells is not due to the presence of an excess of protease inhibitors, nor is it a result of inactivation during the immunoprecipitation process.

Inactivation of the PSA secreted by LNCaP cells could also occur if the majority of the secreted PSA were proteolytically digested by a protease present in the conditioned medium. To confirm whether such a proteolytic inactivation occurs, Western blot analysis of the PSA in the concentrated LNCaP medium was performed (Fig. 2, *Lane 6*). In this analysis, the isolated PSA is found at the correct M_r 33,000 size as expected for intact PSA, and no significant lower molecular weight bands are detected, indicating that the PSA is not being inactivated secondary to significant proteolysis of the PSA protein.

Ability of PSA from LNCaP Cells to Form Complexes with Protease Inhibitors *in Vitro*. PSA in human serum has been shown previously to be enzymatically inactivated by interaction with the extracellular protease inhibitors ACT and A2M (19, 20). These inhibitors are present in 10^4–10^5-fold molar excess to PSA in serum (19). Enzymatically active PSA forms a 1:1 molar ratio complex with ACT by splitting the reactive center of the inhibitor, thus forming a M_r 90,000 stable PSA-ACT covalent complex, which is enzymatically inactive (19). PSA also forms a complex with the high molecular weight A2M by cleaving a peptide bond in the bait region of A2M (20). This cleavage results in a conformational change in the A2M, and as a result, PSA becomes enveloped within the A2M. It has been demonstrated, however, that PSA can be detected in both the PSA/ACT complex form and the PSA/A2M complex form after SDS/heat denaturing and SDS gel electrophoresis (20).

On the basis of the previous assays using the HSSKLQ-AMC substrate, it appeared that <20% of the PSA secreted by LNCaP cells *in vitro* is enzymatically active PSA. To determine whether a portion of the PSA isolated from LNCaP-conditioned medium is enzymatically inactive because of complex formation with ACT secreted by these cells, direct analysis of the medium by Western blot was performed. As seen in Fig. 2 (*Lane 6*) PSA in conditioned serum-free medium from LNCaP is found in the M_r 33,000 uncomplexed form. The addition of excess ACT or A2M to the LNCaP-conditioned medium results in the appearance of either the M_r 90,000 band indicative of the PSA/ACT complex or the M_r 180,000 and M_r >300,000 bands indicative of PSA/A2M complexes (Fig. 2, *Lanes 7* and *8*). Regardless of the inhibitor used, however, most of the PSA is still detectable in the M_r 33,000 uncomplexed form (*i.e.*, >75% using serial dilution standards and densitometric analysis). In contrast, when enzymatically active purified PSA is incubated with these inhibitors, a >90% decrease in the M_r 33,000 band intensity is observed (Fig. 2, *Lanes 2* and *3*). These results are similar to the findings of the enzymatic assays using the HSSKLQ-AMC substrate and confirm that most of the PSA secreted by LNCaP cells is enzymatically inactive.

Activity of Doxorubicin Prodrugs against LNCaP Cells *in Vitro*. Although the above results demonstrate that LNCaP cells produce a low percentage of enzymatically active PSA, this cell line is the only prostate cancer cell line that continues to secrete PSA during serial passage *in vitro*, and, therefore, it was used to test the cytotoxicity of doxorubicin prodrugs. In initial studies, doxorubicin was coupled directly to the

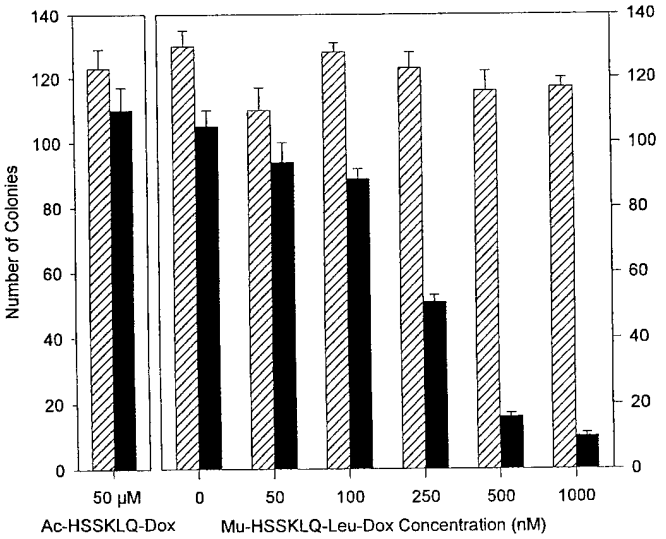


Fig. 4. Clonogenic survival of TSU-Pr1 cells following 48-h treatment with Mu-HSSKLQ-Leu-Dox prodrug with (■) and without (▨) enzymatically active PSA (30 μg/ml). Results are also shown for HSSKLQ-Dox at a 50 μM concentration with (■) and without (▨) PSA (30 μg/ml). Results shown are average (n = 5; *bars*, SE), and assays were done in triplicate.

Ac-HSSKLQ peptide to form the prodrug Ac-HSSKLQ-Dox. Using HPLC detection, it was determined that PSA was unable to hydrolyze the amide bond between the doxorubicin amine and the COOH-terminal glutamine of the peptide (Fig. 3*A*). In medium containing enzymatically active PSA (30 μg/ml), this prodrug had no demonstrable cytotoxicity at concentrations up to 50 μM (Fig. 4).

A previously described doxorubicin derivative consisting of the amino acid L-leucine linked to the primary amine of doxorubicin (designated Leu-Dox) was demonstrated to have activity against cancer cell lines and has been shown to have less cardiac toxicity than doxorubicin in animal models (21, 22). Leu-Dox was found to have a LD_{50} of 50 ± 8 nM against LNCaP cells. Incubation of the Mu-HSSKLQ-Leu-Dox prodrug with enzymatically active PSA resulted in production of Leu-Dox as determined by HPLC, demonstrating that PSA can hydrolyze the glutamine-leucine peptide bond (Fig. 3*B*). After 72-h exposure to PSA-producing LNCaP cells, the Mu-HSSKLQ-Leu-Dox prodrug is >90% hydrolyzed to Leu-Dox (Fig. 3*C*). The Mu-HSSKLQ-Leu-Dox prodrug was then tested against the LNCaP cells and found to be cytotoxic at concentrations as low as 0.1 μM (*i.e.*, 61 ± 2% inhibition at 0.1 μM and 85 ± 2% at 1 μM), with a calculated LD_{50} value of 70 ± 5 nM. These results indicate that LNCaP cells secrete sufficient enzymatically active PSA to release proteolytically the cytotoxic moiety Leu-Dox from the initially inactive Mu-HSSKLQ-Leu-Dox prodrug.

Specificity of the Mu-HSSKLQ-Leu-Dox Prodrug. To document that the toxicity of the Mu-HSSKLQ-Leu-Dox prodrug was dependent on PSA-catalyzed hydrolysis, the cytotoxic response of the TSU-Pr1 PSA-nonproducing human prostate cancer cell line was tested. Unlike LNCaP cells, TSU cells can be used in clonogenic survival assays to accurately determine the cytotoxicity of the Mu-HSSKLQ-Leu-Dox prodrug with and without the addition of enzymatically active seminal plasma-purified PSA to the medium. The LD_{50} for Leu-Dox against TSU cells is 120 ± 4 nM. When enzymatically active PSA was not present, a LD_{50} value for Mu-HSSKLQ-Leu-Dox could not be determined at concentrations up to 1 μM (*i.e.*, clonogenic survival at 1 μM dose not statistically different from control). In contrast, when purified, enzymatically active PSA (*i.e.*, 30 μg/ml) was added to serum-containing medium, the LD_{50} was determined to be 230 ± 5 nM.

Conclusion

These studies demonstrate that PC-82 tumors, when grown as primary cultures *in vitro*, secrete high levels of enzymatically active PSA (*i.e.*, 70 ± 5 ng/10^6 cells/24 h). In contrast, LNCaP cells secrete low levels of enzymatically active PSA (*i.e.*, 2.3 ± 1 ng/10^6 cells/24 h). However, LNCaP cells still secrete sufficient amounts of enzymatically active PSA to activate the Mu-HSSKLQ-Leu-Dox prodrug. The specificity of the cytotoxic response to the Mu-HSSKLQ-Leu-Dox prodrug was documented by the difference in response between the PSA-producing LNCaP cells, in which the LD_{50} value was 70 nM, and PSA nonproducing TSU cells, in which doses as high as 1 μM produce no loss clonogenic ability.

The range of enzymatically active PSA production in the PC-82 and LNCaP cells mimics the situation observed clinically in patients with metastatic prostate cancer. In such patients, metastatic sites are typically composed of prostate cancer cells producing varying amounts of PSA. Previously, it was demonstrated that >90% of the PSA isolated from the extracellular fluid of primary prostate cancers is enzymatically active (11). Once this enzymatically active PSA reaches the blood, it is completely inactivated (11). More than 80% of this inactivation is due to the formation of complexes with ACT and A2M (19, 23). The remaining "free" PSA (*i.e.*, the noncomplexed form) is also not enzymatically active (11). Thus, the PSA-specific prodrug Mu-HSSKLQ-Leu-Dox should not be activated within the circulation. In contrast, once the prodrug diffuses into the extracellular fluid within sites of prostate cancer metastases, it should be hydrolyzed to the active cytotoxin due to the unique presence of high PSA enzymatic activity. Currently, the Mu-HSSKLQ-Leu-Dox prodrug is being synthesized in sufficient quantities to allow *in vivo* studies in LNCaP and PC-82 tumor-bearing mice.

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The Prostate 45:80–83 (2000)

Rapid Communication

In Vivo Activity of a PSA-Activated Doxorubicin Prodrug Against PSA-Producing Human Prostate Cancer Xenografts

Saeed R. Khan and Samuel R. Denmeade*

The Johns Hopkins Oncology Center, The Johns Hopkins School of Medicine, Baltimore, Maryland

BACKGROUND. There is currently no effective therapy for men with metastatic prostate cancer who relapse after androgen ablation. Prolonged administration of effective concentrations of standard chemotherapeutic agents is usually not possible because of dose-limiting systemic toxicities. A new strategy to target cytotoxic agents specifically to sites of metastatic prostate cancer while avoiding systemic toxicity would be to develop prodrugs that are inactive when given systemically but become activated when processed proteolytically within prostate cancer metastases by prostate-specific antigen (PSA). In this study, the in vivo activity of a prodrug consisting of doxorubicin (Dox) conjugated to a PSA-specific peptide carrier is described.

METHODS. Nude mice bearing PSA-producing human prostate cancer xenografts were treated either intraperitoneally (IP) or by continuous infusion with the Dox prodrug. Toxicity (weight loss, death) and antitumor efficacy (tumor volume changes) were determined.

RESULTS. The PSA-peptide Dox prodrug had no discernible systemic toxicity when given at four times the 100% lethal Dox equivalent dose. An IP dose of 60 mg/kg/week × 4 weeks resulted in a 57% decrease in tumor weight vs. control after 40 days. A 25 mg/kg/week dose given by continuous infusion produced a similar decrease in tumor weight vs. control.

CONCLUSIONS. The PSA-specific peptide/doxorubicin prodrug can be used to deliver higher intratumoral levels of Dox for longer duration while avoiding systemic toxicity. In addition, these results validate the specificity of the PSA-specific peptide as a targetable drug carrier. This PSA-specific peptide could also be used as a carrier to target a wide variety of cytotoxic agents for specific activation within sites of metastatic prostate cancer. *Prostate 45:80–83, 2000.* © 2000 Wiley-Liss, Inc.

KEY WORDS: **prostate-specific antigen; prodrug; doxorubicin**

INTRODUCTION

There is currently no effective therapy for men with metastatic prostate cancer who relapse after androgen ablation, even though numerous agents have been tested over the past 30 years [1]. Prolonged administration of effective concentrations of standard chemotherapeutic agents is usually not possible because of dose-limiting systemic toxicities. Therefore, new strategies are needed to target cytotoxic agents specifically to sites of metastatic prostate cancer while avoiding systemic toxicity. One such approach would be to develop prodrugs that are inactive when given systemically but become activated when processed proteolytically within prostate cancer metastases by a prostate-specific protease [2]. To accomplish this, primary amine containing agents can be coupled through the amino group to form a peptide bond to a peptide carrier that is a substrate for a prostate specific protease [2].

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*Correspondence to: Samuel Denmeade, Bunting-Blaustein Cancer Research Building, Rm 1M43, The Johns Hopkins Oncology Center, 1650 Orleans, Baltimore, MD 21231-1001.
E-mail: denmeade@jhmi.edu

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One such primary amine-containing agent that can be readily modified to a prodrug form is the anthracycline doxorubicin. As a single agent, doxorubicin appears to have one of the best response rates from among the large number of agents tested in the treatment of prostate cancer [3] but cardiotoxicity and myelotoxicity have limited its use especially in elderly men. Modifying doxorubicin to an inactive prodrug form may allow prolonged administration with substantially reduced toxicity. To design this prodrug, doxorubicin was linked through its primary amine to a peptide carrier so that the peptide linkage between the cytotoxic agent and the peptide could be specifically cleavable only by the proteolytic activity of prostate-specific antigen (PSA).

PSA is a serine protease with chymotrypsin-like substrate specificity [4–6] that is found in high concentrations (i.e., mg/ml) in the seminal plasma where the major proteolytic substrates for PSA are the gel-forming proteins in freshly ejaculated semen, semenogelin I (Sg I) and semenogelin II (Sg II) [7]. On the basis of the PSA cleavage map for SgI and II, a series of peptides were synthesized and characterized as PSA substrates. From these studies a peptide with the amino acid sequence His-Ser-Ser-Lys-Leu-Gln (HSSKLQ) was identified that had a high degree of specificity for PSA [8]. The HSSKLQ peptide has been coupled to the fluorophore aminomethyl coumarin to produce a fluorescent PSA-specific substrate. This substrate was utilized to demonstrate that prostate cancer cells secrete enzymatically active PSA into the extracellular fluid and that PSA becomes inactivated by serum protease inhibitors upon entering the blood [8].

In a subsequent study, the HSSKLQ peptide was coupled to a doxorubicin analog, *l*-leucinyl doxorubicin (Leu-Dox). Leu-Dox was demonstrated to have activity against cancer cell lines and has been tested in a clinical trial [9]. Leu-Dox was found to be cytotoxic to prostate cancer cell lines with IC_{50} value of 50–100 nM in vitro [10]. The prodrug Mu-HSSKLQ-Leu-Dox was synthesized and PSA hydrolysis to liberate free Leu-Dox was demonstrated [10]. The Mu-HSSKLQ-Leu-Dox prodrug had an IC_{50} against PSA-producing LNCaP cells of 70 nM [10]. Against PSA nonproducing TSU cells, the IC_{50} was > 1 μM while the IC_{50} decreased to 120 nM when enzymatically active PSA was present in the media [10].

On the basis of these in vitro results, sufficient quantities of the Mu-HSSKLQ-Leu-Dox prodrug were synthesized. In the present study the toxicity and antitumor efficacy of the Mu-HSSKLQ-Leu-Dox prodrug was evaluated in nude mice bearing androgen dependent, PSA-producing PC-82 human prostate cancer xenografts.

MATERIALS AND METHODS

Materials

The doxorubicin prodrug Mu-His-Ser-Ser-Lys-Leu-Gln-Leu-Dox (Mu-HSSKLQ-Leu-Dox) where Mu is morpholinocarbonyl was synthesized by coupling the primary amine of doxorubicin to the carboxyl group of the C-terminal amino acid as previously described [10]. Purification by HPLC yielded the trifluoroacetate salt (>98% purity). The peptide sequence was confirmed by amino acid analysis and molecular weights were confirmed by mass spectroscopy. Doxorubicin was from Pharmacia, (Kalamazoo, MI). *l*-leucinyldoxorubicin was a gift from Dr. Attila Nagy, Tulane University, New Orleans, LA.

PC-82 Xenografts

PC-82 is androgen-responsive human prostate cancer xenografts originally established by Hoehn et al. [11]. It was maintained by serial passage in athymic nude mice (Charles River) via an inoculation of 0.2 ml Matrigel containing 20 mg of minced PC-82 tissue as described previously [12]. The enzymatic activity of PSA secreted by PC-82 tumors has been previously characterized. Athymic nude mice were inoculated with PC-82 and tumors measured using a vernier caliper. Tumor volumes were calculated using the formula Width (W) × Length (L) × (W+L)/2 × 0.5236. Animals were injected intraperitoneally (IP) on the first three consecutive days each week. The average tumor size at the start of the IP treatment was ~ 1.8 cc. Tumors were measured twice weekly with calipers and animals sacrificed 40 days after start of treatment. Animals were weighed weekly while on treatment. For continuous infusion of Mu-HSSKLQ-Leu-Dox, animals were anesthetized with Metofane when tumors reached ~0.5 cc in size and an osmotic minipump (Alzet) containing 0.2 ml of Mu-HSSKLQ-Leu-Dox (2.5 mg/ml in distilled H_20) was inserted subcutaneously through a flank incision and closed with staples. The osmotic pump delivered drug at volume of 1 μl/hr equivalent to 25 mg/kg/week. Statistical analysis of differences in tumor volumes and weights between Mu-HSSKLQ-Leu-Dox and vehicle controls were performed using student *t*-test and P values < 0.05 reported in text.

Determination of Mu-HSSKLQ-Leu-Dox Serum Levels

Animals were given 20 mg/kg dose of Mu-HSSKLQ-Leu-Dox IP and at various time points animals were anesthetized and blood obtained by cardiac

puncture. Serum was separated by centrifugation and Mu-HSSKLQ-Leu-Dox extracted by applying 100 μl of serum to a C-18 Bond-Elut column, washing with five column volumes of PBS and then eluting with 70% acetonitrile/0.1% trifluoroacetic acid. Eluent was dried in a speed-vac (Savant) and resuspended in distilled H_20 and absorbance at 480 nm analyzed by HPLC (Beckman). Absolute serum concentrations determined from standard curve using range of concentrations of Mu-HSSKLQ-Leu-Dox.

RESULTS AND DISCUSSION

To be effective the Mu-HSSKLQ-Leu-Dox prodrug should be activated only in the presence of enzymatically active PSA produced within sites of prostate cancer. Little to no nonspecific activation should occur and thus the prodrug should have none of the systemic toxicity of the free drug. To determine whether significant nonspecific systemic activation was occurring when compared to the toxicity of free doxorubicin, animals were treated with equimolar doses of doxorubicin, Leu-Dox or Mu-HSSKLQ-Leu-Dox. One group of animals (n = 5) was given 7 mg/kg (21 mg/week) doxorubicin for 3 consecutive days IP and by day 9, 5/5 animals had died. A second group of animals (n = 3) were given the doxorubicin equivalent (i.e. 9.25 mg/kg or 27.8 mg/week) of leu-dox for 3 consecutive days IP. After the first week, 0/3 animals had died and were therefore given a second 3-day course. At the end of the second week (i.e., 56 mg/kg total Leu-Dox), 3/3 animals had died. For the Mu-HSSKLQ-Leu-Dox group, animals (n = 5) were given 20 mg/kg for 3 consecutive days IP (i.e., 60 mg/kg/week). These animals received a total of four cycles of treatment (i.e., 240 mg/kg total) and one week after the last cycle 5/5 animals were alive. Animals in the Mu-HSSKLQ-Leu-Dox group also experienced no significant weight loss (data not shown).

PC-82 tumor-bearing animals treated with Mu-HSSKLQ-Leu-Dox or vehicle had tumors measured twice weekly. Well-established tumors (i.e., 1–2 cc volume) were utilized in this initial study and there was no significant difference in the average tumor volume between the control and Mu-HSSKLQ-Leu-Dox group at the start of the treatment. The results (Fig. 1) demonstrate a significant decrease in tumor size in the Mu-HSSKLQ-Leu-Dox treated group when compared to vehicle control. The average change in tumor volume ($volume_{day\ 40}/volume_{day\ 0}$) increased 66 ± 18% in the control group and decreased 11 ± 10% in the Mu-HSSKLQ-Leu-Dox treated group, Figure 1 ($P < 0.05$).

At the end of 40 days, these PC-82 tumor-bearing animals were sacrificed and tumor weights obtained. The average tumor weight in the control group was

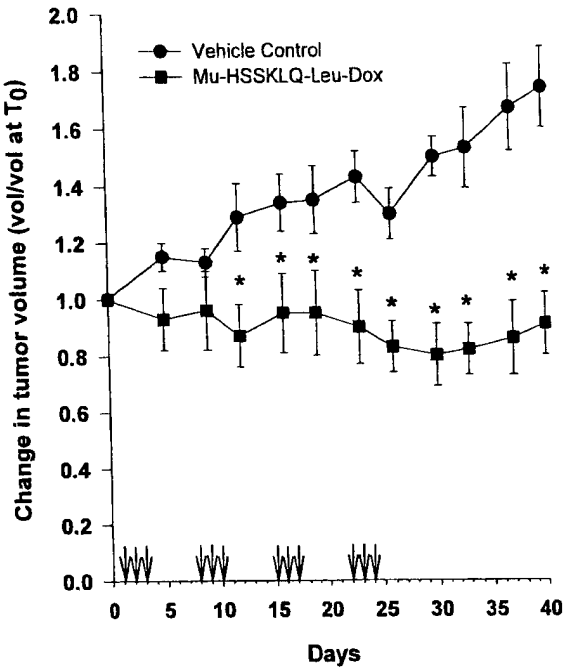

Fig. 1. PC-82 tumor-bearing mice were given 20 mg/kg Mu-HSSKLQ-Leu-Dox intraperitoneally 3 days/week for 4 weeks (dosing indicated by arrows). Tumor volumes were compared to vehicle treated controls with n = 5 animals/group. Change in volume determined by dividing starting tumor volume for each individual animal by volume on day of measurement. Average ± standard error of these volume changes is presented ($P < 0.05$ denoted by stars).

1.97 ± 0.25 g. In contrast the average tumor weight in the Mu-HSSKLQ-Leu-Dox group was 0.85 ± 0.24 g. On the basis of tumor weights, the average tumor weight in the treated group was 57% lower than in the control group ($P < 0.05$). This difference was similar in magnitude to that obtained from the tumor volume measurements.

In the Leu-Dox treated group, 3/3 animals were alive after the first cycle of Leu-Dox therapy. In this group the average tumor volume decreased by 33% on day 11 (i.e., 1.55 ± 0.44 to 1.04 ± 0.20 cc). In contrast, the tumor volume in the control group over a similar 11-day interval increased by 25% (1.87 ± 0.3 to 2.34 ± 0.39 cc) and the tumor volume in the Mu-HSSKLQ-Leu-Dox group decreased by 10% (1.77 ± 0.3 to 1.60 ± 0.4 cc).

To determine whether IP administration of the Mu-HSSKLQ-Leu-Dox resulted in significant serum levels of drug, nontumor-bearing animals were given a single 20 mg/kg dose of Mu-HSSKLQ-Leu-Dox and serum levels were determined at various time points. Peak serum levels of ~ 12 μM were observed 15 minutes post injection and by 24 hr ~90% of the prodrug had been eliminated. By 30 min postinjection intact Mu-HSSKLQ-Leu-Dox was detectable in the urine.

These results suggest that the prodrug readily enters the circulation following IP administration and is rapidly cleared via the kidneys.

This rapid clearance following IP injection suggested that chronic infusion of the Mu-HSSKLQ-Leu-Dox prodrug might be a better method of administration. In a second experiment animals bearing PC-82 tumors were treated with a continuous infusion of the Mu-HSSKLQ-Leu-Dox prodrug via an osmotic minipump. Animals (n = 3/group) received 25 mg/kg total prodrug over 1 week. This dose is ~60% lower than the total weekly dose given IP (i.e., 60 mg/kg). On day 10 following subcutaneous pump insertion, animals were sacrificed and tumor weights obtained. The average tumor weight in the control group was 310 ± 20 mg while in the Mu-HSSKLQ-Leu-Dox treated group the average weight was 170 ± 30 mg. In this study the average tumor weight in the treated group was 46% lower than in the control group ($P < 0.05$). These results demonstrate that a similar antitumor effect can be achieved with 60% less total drug if the prodrug is administered by a continuous infusion.

In summary, these results indicate that the Mu-HSSKLQ-Leu-Dox prodrug can be given systemically with minimal toxicity. The combination of an antitumor effect and lack of systemic toxicity are consistent with specific hydrolysis of the prodrug by enzymatically active PSA within tumors with little to no nonspecific hydrolysis occurring within the blood or other normal tissue. While IP administration of the Mu-HSSKLQ-Leu-Dox prodrug produced a significant antitumor effect in relatively large human tumor xenografts, continuous infusion appears to be the preferred method of prodrug administration. These results suggest that the Mu-HSSKLQ-Leu-Dox could be used to deliver higher intratumoral levels of doxorubicin for longer duration while avoiding systemic toxicity. In addition, these results validate the specificity of the Mu-HSSKLQ peptide as a targetable drug carrier. The lack of significant hydrolysis of the Mu-HSSKLQ-Leu-Dox prodrug presented here suggests that the Mu-HSSKLQ peptide could also be used as a carrier to target a wide variety of cytotoxic agents for specific activation within sites of metastatic prostate cancer. PSA-targeted prodrugs such as Mu-HSSKLQ-Leu-Dox represent a novel therapeutic approach whose further development and testing in clinical trials is warranted.

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BIOORGANIC & MEDICINAL CHEMISTRY

Thapsigargin Analogues for Targeting Programmed Death of Androgen-Independent Prostate Cancer Cells

S. Brøgger Christensen,[a,*] Annette Andersen[a], Hasse Kromann,[a] Marek Treiman,[b] Bertrand Tombal,[c] Sam Denmeade[c] and John T. Isaacs[c]

[a]*Department of Medicinal Chemistry, Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark*
[b]*Department of Medical Physiology-Biotechnology Center for Cellular Communication, University of Copenhagen, The Panum Institute, Blegdamsvej 3C, DK-2200 Copenhagen N, Denmark*
[c]*Johns Hopkins Oncology Center, Johns Hopkins School of Medicine, 422 N. Bond Street, Baltimore, MD 21231, USA*

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Abstract—A number of analogues of thapsigargin, a selective inhibitor of the sarco-endoplasmic reticulum Ca^{2+}-ATPases have been synthesized. In all of the prepared analogues the butanoyl residue at *O*-8 has been replaced with a residue containing an aromatic amine. The amine can be used as an anchoring point for attaching a peptide group sensitive to the proteolytic enzyme, prostate specific antigen, secreted by prostate cancer cells. Like thapsigargin, the analogues are capable of elevating the cytoplasmic Ca^{2+} concentration approximately sevenfold when tested at effective cytotoxic doses. The analogues in which the 8-*O*-butanoyl group has been replaced with 3-(4-aminophenyl)propanoyl or 4-aminocinnamoyl were found potently to induce programmed cell death of the prostate cancer cells.

Introduction

Currently there is no treatment that significantly prolongs survival in men with metastatic prostate cancer.[1] Androgen ablation therapy eventually fails because the metastatic prostate cancer within an individual patient is heterogenously composed of clones of both androgen dependent and independent cancer cells.[2] Thapsigargin (**1**, Tg, Fig. 1) is a sesquiterpene lactone[3] that selectively inhibits the sarcoplasmic and the endoplasmic reticulum Ca^{2+}-ATPases (SERCA).[4,5] Inhibition of the SERCA causes depletion of intracellular Ca^{2+} stores resulting in an initial rise in cytoplasmic Ca^{2+} concentration, which by capacitance influx of extracellular Ca^{2+} affords a secondary sustained elevation of cytoplasmic Ca^{2+}. This sustained elevation in the cytosolic Ca^{2+} concentration activates the proliferation independent programmed death of susceptible cells including androgen independent prostate cancer cells.[6] Since the SERCA is present in almost all cells Tg will also induce apoptosis in many normal host cells. Consequently a method of targeting the proliferation independent cytotoxicity of Tg selectively to prostate cancer cells is needed. In order to target Tg, advantage can be taken of the secretion of prostate-specific antigen (PSA) by prostate cancer cells.[7] PSA is a serine protease with an unusual specificity. Thus, PSA efficiently liberates 7-amino-4-methylcoumarin from a substrate in which the amino group of this coumarin is coupled to the six-amino acid peptide His-Ser-Ser-Lys-Leu-Gln. In contrast, this conjugate is a very poor substrate for other purified proteases and proteases present in sera.[8] The specificity has further been illustrated by incubating cancer cells with a prodrug in which the amino group of doxorubicin is coupled to this six-amino acid. Only PSA producing cells are killed, indicating that doxorubicin is only liberated in the vicinity of such cells.[9] A fast inactivation of PSA outside the prostate ductal system means that the hydrolytic activity only will be of significance in the close vicinity of prostate cells and prostate cancer cells. The aim of the present project is to develop a prodrug consisting of a primary amine containing Tg analogue, which via the amine group is coupled to a PSA cleavable peptide.[3] This paper describes design, synthesis and SERCA inhibitory potencies of analogues of Tg that can be coupled to promoiety groups.

Key words: Prostate cancer; prodrug; thapsigargin; prostate-specific antigen; apoptosis.
* Corresponding author. Tel.: +45-3537-0850; fax: +45-3537-2209; e-mail: sbc@mail.dfh.dk

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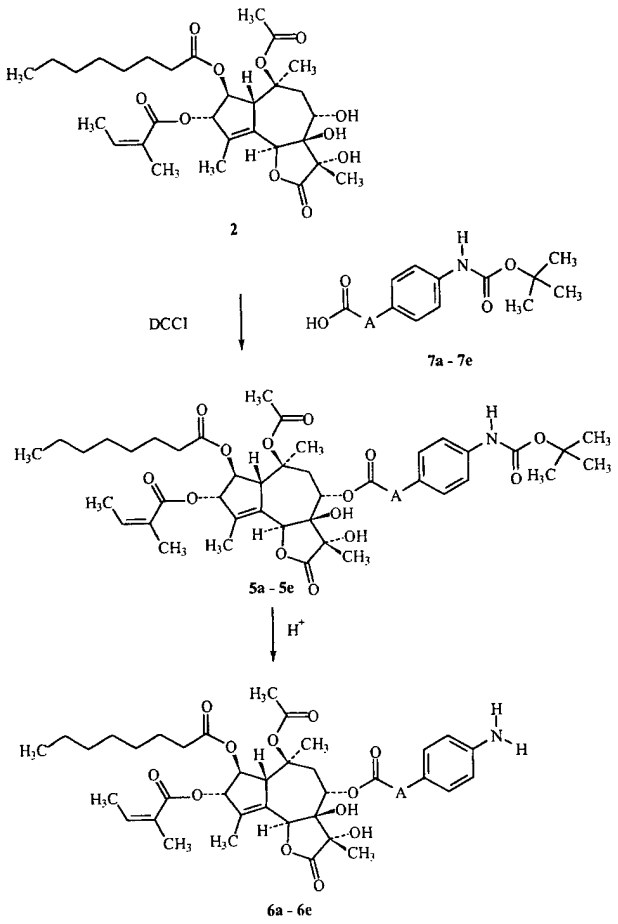
as that of Tg. Surprisingly the LC_{50} value of **6a** was 100 times less active than Tg and **6b** only showed minor activity in concentrations up to 10 μM.



a, A = -$(CH_2)_0$-; b, A = -(CH_2)-, c; A = -$(CH_2)_2$; d, A = -CH=CH-; e, A = -$(CH_2)_3$-

Scheme 2. Synthesis of the analogues containing a 4-aminobenzene carboxylic acid (**5a–5e** and **6a–6e**).

The cytotoxicity of Tg on prostate cancer cells is due to its ability to sustain an increase of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) more than 10 h leading to the subsequent activation of the programmed death pathway in these cells.[6] Several of the analogues were tested for their ability to elevate $[Ca^{2+}]_i$ in TSU cells using appropriate drug levels as based upon their LC_{50} values. The 4 analogues **4a** and **6c–6e** produced the same initial rise in $[Ca^{2+}]_i$ as Tg and also like Tg sustained an approximately sevenfold elevation in $[Ca^{2+}]_i$ for more than 4 h of treatment in TSU cells, Table 2. If EGTA was added to lower the extracellular Ca^{2+} concentration to below 100 nM in the media, then the $[Ca^{2+}]_i$ levels rapidly (i.e. minutes) return to below 50 nM. After 4 h of exposure to Tg or the analogue, further addition of 1 μM Tg to the media did not result in any further increase in $[Ca^{2+}]_i$. This indicates that theses analogues like Tg itself [6] deplete the ER calcium stores leading to the capacitive entrance of extracellular Ca^{2+} into the cells to elevate $[Ca^{2+}]_i$.[11] The analogue **6b** that did not induce programmed cell death was also unable to increase the $[Ca^{2+}]_i$ up to a concentration of 10 μM, Table 2.

In conclusion, coupling of the analogues **6c–6e** to peptides, which are selectively cleaved by PSA will allow for targeted delivery of these cytotoxic agents to sites of PSA producing metastatic prostate cancer. Currently we are coupling the analogues to the peptide carrier for further in vitro and in vivo testing for activity against PSA producing prostate cancers.

Experimental

Chemistry

The spectra have been recorded on a AF200X Bruker spectrometer in deuterated chloroform solutions using tetramethylsilane as an internal standard. In all the spectra the signals originating in the acetyl, angeloyl,

Table 1. IC_{50} values for inhibition of the skeletal muscle Ca^{2+}-ATPase and LC_{50} values for loss of clonogenic survival for Tg (**1**) and some analogues against TSU-Prl human prostate cancer cells

Compound	To inhibit Ca^{2+}-ATPase 50%[a] (IC_{50} in nM)	To inhibit Ca^{2+}-ATPase 50%[b] (IC_{50} in nM)	Activity relative to Tg (**1**)[c]	To kill 50% of clonogenic cells (LC_{50} in μM)
Tg (**1**)	3.9 ± 0.2 (6)	11.0 ± 1.1 (4)	1	0.030 ± 0.004 (5)
dBTg (**2**)	21 ± 1 (3)		0.19	10.0 ± 0.5 (5)
4a sch1	17.2 ± 2.4 (3)		0.22	3.1 ± 0.2 (5)
4b sch1	316 ± 22 (3)		0.012	16.6 ± 0.4 (5)
4c sch1	1278 ± 91 (3)		0.0030	> 25
6a sch2		18.4 ± 0.3 (3)	0.59	4.0 ± 0.3 (5)
6b sch2		n.d.		> 10
5c sch2		21.2 ± 0.8 (3)	0.52	0.87 ± 0.02 (5)
6c sch2		16.2 ± 0.9 (3)	0.68	0.28 ± 0.06 (5)
5d sch2		20.3 ± 0.2 (3)	0.54	1.9 ± 0.1 (5)
6d sch2		18.7 ± 0.2 (3)	0.59	0.11 ± 0.03 (5)
5e sch2		14.4 ± 0.8 (3)	0.76	n.d.
6e sch2		18.4 ± 1.4	0.60	0.23 ± 0.02 (5)

Results expressed as mean ± standard error (number of experiments).
[a] The concentration of SR protein in the test solution was 2 μg/mL.
[b] The concentration of SR protein in the test solution was 5 μg/mL.
[c] The ratio of the IC_{50} value of Tg/IC_{50} value of the analogue.

which increasing amounts of ethyl acetate were added as an eluent. ^{1}H NMR ($CDCl_3$) δ guaianolide 4.25 (br.s, H-1), 5.68 (br.s, H-6), 5.63 (br.s. H-8), 5.59 (t, J=3 Hz, H-3), 5.47 (br.t, J=2 Hz, H-2), 2.97 (dd, J=20 and 3 Hz, H-9), 1.84 (br.s H-15), 1.40 (s, H-13), 1.37 (s, H-14); aminophenylbutanoyl 2.52 (t, J=9 Hz, H-α), 2.24 (t, J=9 Hz, H-γ), 1.80 (m, H-β), 6.94 (d, J=7 Hz, H-2 and H-6), 6.64 (H-3 and H-5); ^{13}C NMR ($CDCl_3$) δ guaianolide 57.5 (C-1), 76.7 (C-2), 84.1 (C-3), 141.6 (C-4), 130.5 (C-5), 77.8 (C-6), 78.5 (C-7), 66.1 (C-8), 38.1 (C-9), 84.6 (C-10), 78.5 (C-11), 178.5 (C-12), 15.7 (C-13), 22.5 (C-14), 12.8 (C-15); 4-aminophenylbutanoyl 172.7 (C=O), 34.0 (C-α), 33.6 (C-γ), 26.9 (C-β), 129.3 (C-1), 129.3 (C-2 and C-6), 115.7 (C-3 and C-5), 143.9 (C-4). MS FAB$^-$ Found 740.3672. Calc. for $C_{40}H_{55}NO_{12}$ 740.3646.

Pharmacology

SERCA assay. Sarcoplasmic reticulum (SR) vesicles from rabbit skeletal muscle were prepared according to De Meis and Hasselbach[14] and stored in *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) 20 mM (pH 7.0), sucrose 300 mM, at −80 °C.

ATPase activity was measured as $^{45}Ca^{2+}$ uptake to SR vesicles at 25 °C, pH 7.0, using 2–5 μg/mL of protein. Triplicate samples of SR membranes were preincubated for 10 min in the uptake medium minus ATP, in the presence of the desired inhibitor concentrations (added as 100- or 200-fold concentrated stock solutions in DMSO; corresponding amount of DMSO was present in control incubations). The medium composition was then completed by adding ATP (or TES buffer for the blank determinations) to start the uptake reaction. The complete medium contained (concentrations in mM): TES 20; sucrose, 60; KCl 110; $MgCl_2$ 5; K^+-oxalate 6; EGTA 1; $^{45}CaCl_2$ 0.2 (about 2 μCi/mL); ATP 2.5 mM. (The concentration of free Ca^{2+} ions was 0.1 μM, calculated according to Foehr et al. 1993[15]). The uptake was stopped after 4 min by filtration through Whatman GF/F filters (presoaked in 0.1% polyethyleneimine to reduce the background radioactivity) on a Brandel Harvester M24 apparatus (SEMAT Technical Ltd., UK). The filters were washed immediately in an ice-cold buffer containing (concentrations in mM): tris/HCl 20 (pH 7); NaCl 140; $MgCl_2$ 10; and counted using Opti-Fluor scintillation liquid (Packard Instruments, DK) at 100% efficiency. The time course of the uptake was linear at least up to 4 min.

The reported IC_{50} values are means of the numbers obtained from 4-parameter logistic equation fitting (GRAFIT program, Erithacus Software Ltd.) of the doses–response data, with the number of individual experiments indicated for each compound. For thapsigargin derivatives, these values are reported relative to that of thapsigargin when a corresponding amount of SR protein was used. This normalization of the thapsigargin analogue IC_{50} values to those of thapsigargin was necessary because of the stoichiometric nature of thapsigargin binding to Ca^{2+}-ATPase, resulting in shifts of the apparent inhibitory potency in parallel with the concentration of the enzyme.[16]

Determination of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). TSU cells (10^6) were treated with the indicated analogues for 1 h at 37 °C. Cells were then loaded with Fura 2-acetoxymethyl ester (5 μM) (0.1% DMSO/0.001% Plutonic) (Molecular Probes, Eugene OR) for 30 min at 33 °C in phenol red free RPMI media. Cells were then pelleted and resuspended in buffer of (4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES)-Krebb's medium [108 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 11.5 mM glucose, and 10 mM HEPES-KOH (pH 7.4)]) for an additional 20 min. Cells were then repelleted and washed just prior to assay then suspended in a quartz cuvette and fluorescence emission at 510 nm was measured after excitation at 340 nm and 380 nm using a photomultiplier from PTI (New Brunswick, NJ) at 25 °C. $[Ca^{2+}]_i$ were calculated from ratios from fluorescence intensities obtained every 1 second using the calculations of Grynkiewicz et al. and a K_d of 150 nM.[17] Dye was considered saturated on addition of 10 μM ionomycin while the minimum fluorescence ratio was determined in the presence of 5 mM EGTA.

Cytotoxicity assays. Percentage clonogenic survival of TSU-Pr1 (2×10^5 cells) following 2 h exposure to varying concentrations of Tg analogues was performed as described previously.[18]

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